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Toxicity, bioaccumulation and biotransformation of Cu oxide nanoparticles in *Daphnia magna*†

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This study investigated the toxicity, bioaccumulation and biotransformation of copper oxide nanoparticles (nCuO) and $CuSO_4$ in *Daphnia magna*. We performed acute and chronic assays, and analyzed the organisms by μ-XRF and μ-XANES. In acute assays 25 nm nCuO (LC_{50} 0.05 ± 0.011 mg Cu per L) and $CuSO_4$ (LC_{50} 0.16 ± 0.015 mg Cu per L) were most toxic, while 40 nm and 80 nm nCuO had similar toxicity (LC_{50} 2.34 ± 0.479 and 2.26 ± 0.246 mg Cu per L, respectively). In chronic assays, $CuSO_4$ (EC_{50} 1.7×10^{-4} ± 1.0×10^{-4} mg Cu per L) was most toxic followed by 25 nm nCuO (EC_{50} 1.8×10^{-3} ± 8.0×10^{-4} mg Cu per L), while 40 and 80 nm nCuO were least toxic (EC_{50} 2.10 ± 0.669 and 1.95 ± 0.568 mg Cu per L, respectively). μ-XRF showed that Cu was accumulated in the intestine and appendages of the daphnids. μ-XANES showed that 25 nm nCuO and $CuSO_4$ were biotransformed into $Cu_3(PO_4)_2$ (acute assays), whereas 40 and 80 nm nCuO remained as CuO (chronic assays). The higher toxicity exhibited by $CuSO_4$ and 25 nm nCuO can be explained from their higher chemical reactivity (probed by catalytic decomposition of H_2O_2 and μ-XANES) compared to 40 and 80 nm nCuO.

Environmental significance

Nanomaterials properties such as size dependent solubility, light absorption and surface reactivity make them promising players towards the development of disruptive technologies in crop science. In this context, Cu based nanomaterials might simultaneously act as fungicide and fertilizer. However, after field broadcasting part of these nanomaterials may run off and end up in water streams. Therefore is paramount to assess the effects of nanomaterials on aquatic living organisms, and well as to understand the mechanisms behind toxic effects. To accomplish such important task, this study combined toxicity assays on *Daphina magna* model organism with cutting edge X-ray spectroscopy techniques.

Introduction

Pesticides and fertilizers have been of major importance for meeting the demand for food, feed and biomass. However, their indiscriminate use can lead to soil, water and crop contamination.

Copper is an essential element for plants,[1,2] its concentration in vegetal tissues ranges from 1–5 mg Cu per kg dry mass.[3] Due to the conversion of Cu^{2+} to Cu^{+} and *vice versa*, this element plays key roles in the photosynthetic electron transport chain, in respiration and as a cofactor of enzymes.[4] Since Cu is exported from fields after each crop harvest, it must be resupplied as fertilizer. This keeps its adequate level in the soil without decreasing crop yield. The most common chemical forms in Cu-containing fertilizers are EDTA chelates, sulphates and oxides.[5,6] Cu-based compounds, such as oxides, hydroxides, sulphates, carbonates and organic complexes, are also employed as pesticides for controlling weeds, mollusks, algae, bacteria and fungi.[7–9]

Nanotechnology-based products ready for use as fertilizer or pesticide can be found on the shelf of stores.[10] Moreover, the inventory of the 'Project on Emerging Nanotechnologies', which has been founded in the United States and since 2005 provides information on nanotechnologies, lists 10 products containing copper nanomaterials in water filter cartridges, food supplements and skin care products.[11] The world production of copper oxide nanoparticles (nCuO) is expected to be 1600 tons by 2025.[7]

In agriculture, the application of nCuO was able to increase plant growth,[12,13] improve seedling weight gain and

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reduce stress.[14] Copper oxide nanoparticles have also been applied in catalysts,[15] photodetectors,[16] biosensors[17] and batteries.[18] A common problem of all these types of usage is the fact that nCuO might eventually reach the environment such as soil and water streams.

The literature reports on the acute and chronic toxicity of nanoparticles to aquatic organisms like bacteria,[19] algae,[20] plants,[21] crustaceans[22,23] and fish.[20,24] However, few studies report on the spatial distribution and chemical speciation of the nanoparticles or released metal ions inside the test organisms. This type of information could help to understand the causes of toxicity.

Currently, most studies on *Daphnia magna* indicate oxidative stress[22,25–31] and dissolved copper[22,25–29] as the main causes of toxicity in this organism. Reactive oxygen species can be formed by chemical species, such as Cu^{2+}, which causes oxidative stress in the organism.[22] This stress can cause damage to lipids, carbohydrates, proteins and DNA.[22,26] Radical species can impair the cell membrane and lead to loss of cellular functions.[26] Other studies have reported a relationship of the toxicity of nanomaterials with particle size.[30] The daphnids can absorb particles[30] which can block the gastrointestinal tract resulting in malnutrition.[31]

X-ray fluorescence microanalysis (μ-XRF) combined with microprobe near edge X-ray absorption spectroscopy (μ-XANES) are non-destructive analytical tools that can reveal the elemental spatial distribution of a nanomaterial and its chemical environment in biological tissues.

The present study aimed at evaluating and comparing the toxicity of commercially available copper oxide particles (25, 40 and 80 nm, according to the supplier) to *D. magna*. Acute and chronic assays were performed to assess the LC_{50} for effects on survival and EC_{50} for effects on reproduction, respectively. In addition, we employed μ-XRF and μ-XANES to understand how the internal distribution of the Cu and its chemical speciation could help explaining nCuO toxicity to the daphnids.

Experimental

Nanoparticle and dispersion characterization

Three commercial Cu oxide based nanoparticles were employed in this study, having nominal sizes of 25, 40 and 80 nm (US Nanomaterials Research Inc). The size and shape of the nCuO nanoparticles were characterized in dispersions at 10 mg Cu per L by transmission electron microscopy (TEM) (JEM-1011, Carl Zeiss AG, Germany). The dispersions were sonicated with a Sonic Dismembrator (Model 705, Fisher Scientific, USA) at 95 W, amplitude of 50% and 50 J for 4 cycles of 5 min each and intervals of 3 min between cycles. The crystal structure of the nCuO was determined by Cu-Kα radiation X-ray diffraction (XRD) using a PM 1877 diffractometer (Philips, Netherlands).

To estimate the solubility of nCuO, we prepared 50 mL aqueous dispersions in deionized water and daphnid culture medium with the 25, 40 and 80 nm nCuO particles at 100 mg Cu per L. A solution of $CuSO_4$ at the same concentration was also prepared for comparison. The time and parameters of the sonication were the same for TEM, except that the dispersions were kept at room temperature for 24 h. Then, 1 mL of each dispersion was centrifuged using a microcentrifuge (Mikro120, Hettich, Germany) for 1 h at 13 000 rpm. The supernatants were collected and analyzed by energy dispersive X-ray fluorescence (EDXRF) (EDX-720, Shimadzu, Japan). The quantification was performed using a calibration curve with an internal Ga standard under thin film conditions (see the ESI,† for details).

For zeta potential and dynamic light scattering (DLS) analysis, dispersions of nCuO at 100 mg Cu per L were prepared as describe above. The measurements were carried out in water and culture medium (without algae) using a Zetasizer Nano (Malvern Instruments, U.K).

Geochem[32] was employed to assess Cu chemical species in water and culture medium. The input concentrations and pH are presented in the ESI.†

Toxicity assessments

Experimental conditions. *Daphnia magna* neonates were grown in 2 L beakers stored in an incubator at 22 ± 1 °C and 12 hours photoperiod. The culture medium was prepared with deionized water at pH 7–7.5, according to ABNT 12713 (2016).[33] The daphnids were fed three times a week with suspensions of *Raphidocelis subcapitata* (density 10^6 cells per mL) and trout feed solution (5 g L^{-1}). The medium was changed three times per week.

Preparation of dispersions and dilution for acute and chronic assays

Three stock dispersions of 25, 40 and 80 nm nCuO and a stock solution of $CuSO_4{\cdot}7H_2O$ (P.A. Synth), both at 50 mg Cu per L, were prepared in deionized water. Then, the dispersions were sonicated as described above.

Sensitivity, acute and chronic assays

Prior to the acute and chronic assays, sensitivity assays were performed with the reference substance NaCl, exposing neonates to concentrations of 1, 3, 5 and 7 g L^{-1}. Sensitivity and acute assays were performed with five neonates (≤24 hold) per replicate. The acute assays with nCuO were performed according to ABNT (2016).[33] $CuSO_4$ was used as positive control and culture medium as negative control. The concentrations of nCuO and $CuSO_4$ in the culture medium ranged from 0.015 to 16 mg Cu per L, using five test concentrations (see Table S1 in the ESI†). The daphnids were exposed in polyethylene flasks containing 30 mL of test solution and kept in an incubator at 22 ± 1 °C and 12 h photoperiod for 48 h. During the exposures, no precipitation of nCuO was observed on the bottom of the flasks. After this period, the dead or immobile individuals were counted. The Priprobit method

was used to calculate the concentration that killed or immobilized 50% of the organisms (LC_{50}).[34]

The chronic assays were performed according to OECD (1998).[35] Test concentrations ranged from 1.6×10^{-4} to 1.9 mg Cu per L, using four concentrations in culture medium (see Table S2 in the ESI†), which were chosen based on preliminary tests. Neonates, less than 24 h old, were exposed individually in polyethylene flasks containing 100 mL culture medium spiked with nCuO dispersions or $CuSO_4$ solution. For each treatment and negative control (culture medium), 10 replicates were prepared and maintained at 22 ± 1 °C with photoperiod of 12 hours. Treatment solutions, culture medium and feed (*R. subcapitata*, at 10^3 cells per mL) were replaced three times per week, and tests lasted for 21 days. Every day the numbers of new-borns were assessed. At the end of the assays, the averages of posture and reproduction were calculated as described in the ESI.†

Decomposition of H_2O_2 by nCuO and $CuSO_4$

To assess the surface reactivity of the nCuO, the decomposition rate of H_2O_2 was evaluated in terms of the volume of O_2 generated. For this, 19.5 mL of a 1000 mg Cu per L dispersion of nCuO or a solution of $CuSO_4$ was prepared in deionized water and in culture medium. Similar solutions were prepared in culture medium at concentrations corresponding to the LC_{50}s. For 1000 mg Cu per L, the samples were placed in a 25 mL sealed flask, connected to a syringe and to a 25 mL pipette placed in the water column. Then, 0.5 mL of H_2O_2 30% v/v was added and the volume of O_2 produced was measured every five minutes for 300 minutes or until it exceeded the graduation limits of the 25 mL pipette. The tests were repeated twice. The same procedure was applied three times for concentrations corresponding with the LC_{50} using a 5 mL pipette for 48 h.

X-ray fluorescence microanalysis (μ-XRF)

μ-XRF was employed to map the 2D spatial distribution of Cu taken up by the daphnids that died upon exposure to nCuO and $CuSO_4$. This allowed uncovering the Cu location at the moment of death. The daphnids were removed from the test media, washed three times with phosphate buffer (PBS) (Na_2HPO_4, $NaH_2PO_4.H_2O$, pH 7–7.5) and fixated in 4% paraformaldehyde solution (PFA) overnight, washed again three times with PBS and kept under PBS at 4 °C till analysis.

The daphnids were taken from the PBS with a pipette and transferred to the top of a Kapton™ (polyamide) thin film mounted on an XRF cuvette (see Fig. S1(A) in the ESI†). The Cu spatial distribution was determined using a benchtop μ-XRF system (EDAX, Orbis PC, USA). X-rays were generated by a Rh anode. The 30 μm X-ray beam (for the Mo-Kα) was focused on the samples by poly-capillary optics, and the detection was carried out by a 30 mm^2 silicon drift detector operating at 140 eV resolution for Mn-Kα. The maps were recorded using a matrix of 64 × 50 points summing up to 3200 XRF spectra for each image. The maps for daphnids from the acute assays were acquired using 40 kV, 300 μA and 1.5 s dwell time per point while the maps for daphnids from the chronic assays were registered under 50 kV, 800 μA and 6 s dwell time per point. The latter measurements were performed using a 25 μm Ti primary filter. All maps were recorded under a dead time lower than 3%.

The Cu instrumental sensitivity was determined using a CuS thin film Micromatter™ standard containing 42.3 μg Cu per cm^2 (serial #6323). X-ray transmission assays (not shown here) revealed that the neonates are infinitely thin samples for Cu Kα radiation. Then, Cu quantification was performed by dividing the number of XRF Cu-Kα counts (cps) by the elemental sensitivity (cps μg^{-1} cm^{-2}). The thickness of adult *D. magna* did not allow performing quantitative analyses and therefore only qualitative Cu maps will be presented.

Microprobe X-ray absorption near edge spectroscopy (μ-XANES)

Cu-K edge μ-XANES spectra were recorded at the XRF beamline of the 1.37 GeV Brazilian Synchrotron Light Laboratory (LNLS, Campinas). In this facility X-rays were provided by a bending magnet device, monochromatized by a double crystal Si(111), and the 20 × 25 μm^2 X-ray beam was focused on the sample by a KB mirror system. The detection was carried out in XRF mode using an element Si drift detector (KETEK GmbH, Germany). At least six μ-XANES spectra were recorded per sample, each of them recorded in *ca.* 40 min in the same position. The energy step in the edge region was 0.5 eV. The spectra were subsequently merged to improve the signal to noise ratio.

Chemical distribution maps previously recorded in our laboratory helped to define the appropriate regions of the daphnids to be measured by μ-XANES. The spots with higher Cu content were analysed. The chemically fixed samples (same procedure as described for μ-XRF) were selected for these analyses. To prevent dehydration, the samples were covered with a 4 μm Ultralene™ (polyethylene) film (see Fig. S1(B) of the ESI†).

In addition to these samples, Cu reference compounds previously synthesized in our laboratory were measured. The complexation procedure was similar to that for Zn reported by Sarret *et al.*[36] The mixtures of aqueous $Cu(NO_3)_2$ and salts at pH 5 were stirred for 24 h and freeze dried. The μ-XANES spectra were energy calibrated using a reference Cu foil. The data was normalized using the Athena software of the IFEFFIT package.[37]

Results and discussion

Characterization of nCuO

X-ray diffraction patterns (Fig. S2†) showed that the 40 and 80 nm CuO particles were monoclinic CuO, the 25 nm nCuO particles contained a fraction of face centric cubic (fcc) metallic phase in addition to monoclinic CuO. In a previous study we showed that the 25 nm CuO particles had a core-shell structure.[38]

The average particle sizes determined by TEM (Fig. S3†) were close to the values reported by the supplier. nCuO 25 nm (26 ± 8 nm) were of spherical shape, 40 nm (45 ± 11 nm) of elliptical shape and 80 nm (75 ± 19 nm) of quadratic shape. Nevertheless, for the sake of clarity, we decided to keep the nominal size mentioned by the supplier when reporting the results obtained.

For reasons of feasibility, DLS and zeta potential measurements (Table 1) were performed using concentrations above those used in the bioassays. The dispersed particles aggregated. In agreement with a previous study, the hydrodynamic diameters in culture medium were larger than those recently reported for dispersions in deionized water.[39] This is partially explained by the decrease in zeta potential, which ultimately leads to lower electric repulsion between the particles. Other studies also reported aggregate size and zeta potentials in the same orders of magnitude (−17.6 mV, −9.6 mV and 935.5 nm 1095 nm, respectively).[25,40]

Table 1 shows that in water and culture media, Cu solubility was inversely proportional to nanoparticle size. This trend is in agreement with the literature.[39] The solubility of $CuSO_4$ was higher in deionized water than in culture medium, which suggests that part of the Cu^{2+} ions had precipitated. Calculations performed by Geochem[32] showed that 85% of the Cu was precipitated with OH^- in the 100 mg Cu per L solution (Fig. S4†). Thus, when interpreting the dose–response curves one should keep in mind that a significant fraction of the putative active free or ionic Cu may actually be precipitated.

Acute and chronic assays

The acute assays revealed the concentrations necessary to kill 50% of the daphnid population within 48 hours exposure. The chronic assays focused on the effects of low concentrations during a long-term exposure. Therefore, these tests are complementary.

Fig. S5† shows the sensitivity of *D. magna* neonates to the reference substance NaCl. The LC_{50} of 4370 mg L^{-1} was close to the values of 4868 mg L^{-1} and 4765 mg L^{-1} reported in the literature.[41,42] This confirms that the organisms used were in good condition.

Fig. 1 shows the mortality (dose–response curve) of the daphnids exposed to 25, 40 and 80 nm nCuO and the $CuSO_4$ positive control. The 25 nm CuO and $CuSO_4$ were the most toxic with LC_{50}s of 0.05 ± 0.011 mg Cu per L and 0.16 ± 0.015 mg Cu per L, respectively. The LC_{50} values for 40 and 80 nm nCuO were similar at 2.34 ± 0.0479 and 2.26 ± 0.246 mg Cu per L, respectively. According to the statistical analysis presented in Fig. 2, the order of acute toxicity was: 25 nm CuO = $CuSO_4$ > 40 nm nCuO = 80 nm.

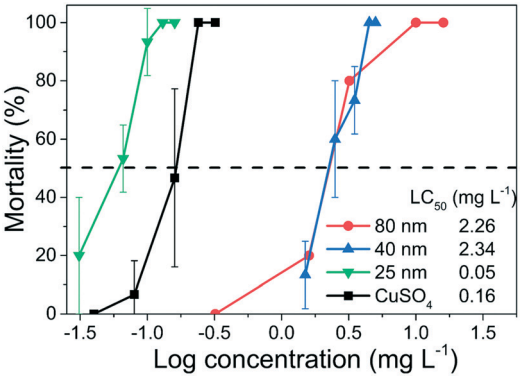

Fig. 1 Dose–response curves for the acute toxicity of nCuO and $CuSO_4$ to *Daphnia magna* neonates.

The calculation performed by the Geochem code showed that in the 0.16 mg Cu per L $CuSO_4$ solution most of the copper was coordinated as CuEDTA. Thus, if the toxic effect was caused by free Cu^{2+} ions, the actual Cu ionic concentration corresponded to nearly one tenth of that supplied as $CuSO_4$. This means that the Cu ionic toxic threshold is actually lower than supposed.

Table S3† compiles the LC_{50} values found by other research groups evaluating the acute toxicity of nCuO to *D. magna*. According to Table S3,† nCuO smaller than 100 nm yielded LC_{50} values from 0.08 to 4.0 mg Cu per L while LC_{50}s for $CuSO_4$ ranged from 0.04 to 0.17 mg Cu per L. The $CuSO_4$ LC_{50} reported in the present study is in close agreement with ref. 22 and 26 however, it strongly differs from ref. 30.

Although the values reported in Table S3† give an idea of the toxicity of Cu salts and nCuO, it is difficult to compare the LC_{50} data generated by these studies. In spite of recommendations in the test guidelines,[43–45] some of the above mentioned studies[27,29,31,46] did not determine the sensitivity of the daphnids to a reference substance, *e.g.* NaCl. Therefore, to facilitate data comparison future studies should also report this parameter.

In principle, the observed toxicity to neonates may relate to an imbalance of dissolved ions, channel obstruction or reactions taking place on the surface of the particles. Previous

Table 1 Physical–chemical characterization of the treatments of nCuO and $CuSO_4$

	Hydrodynamic diameter (nm)		Zeta potential (mV)		Dissolved Cu (mg L^{-1})	
Treatment	dw	cm	dw	cm	dw	cm
25 nm	263 ± 3	819 ± 353	16 ± 0.1	−10 ± 0.2	1.0 ± 0.3	1.0 ± 0.7
40 nm	153 ± 45	171 ± 91	−20 ± 0.6	−8 ± 0.7	0.6 ± 0.2	0.1 ± 0.06
80 nm	214 ± 3	292 ± 3	−17 ± 0.2	−11.8 ± 0.2	0.2 ± 0.01	0.3 ± 0.3
$CuSO_4$	—	—	—	—	117 ± 10	40 ± 7

dw = deionized water; cm = culture medium.

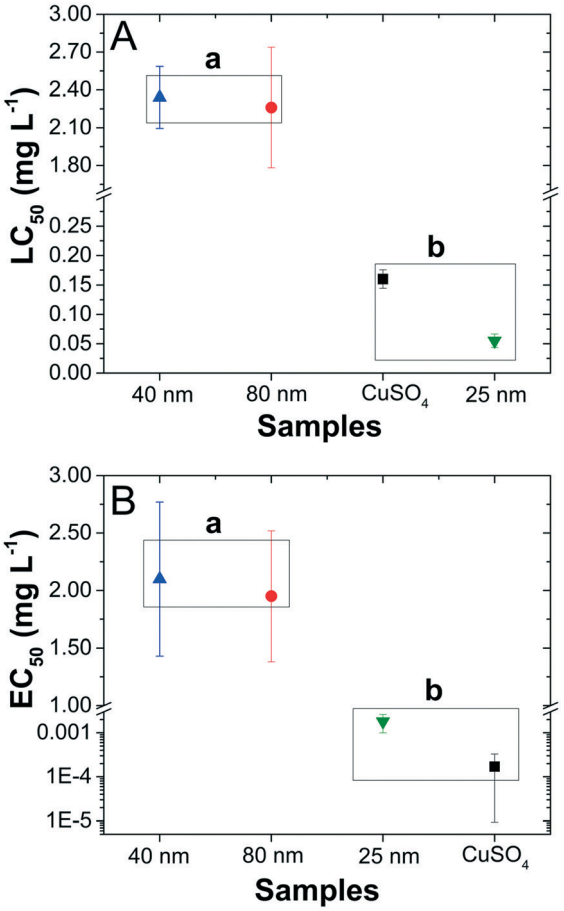

Fig. 2 Effects of nCuO and $CuSO_4$ on survival and sublethal endpoint in assays with *Daphnia magna*. Comparison of (A) LC_{50} values from acute assays and (B) EC_{50} values from chronic assays. Values followed with the same indices do not differ significantly according to the Tukey test ($p < 0.05$).

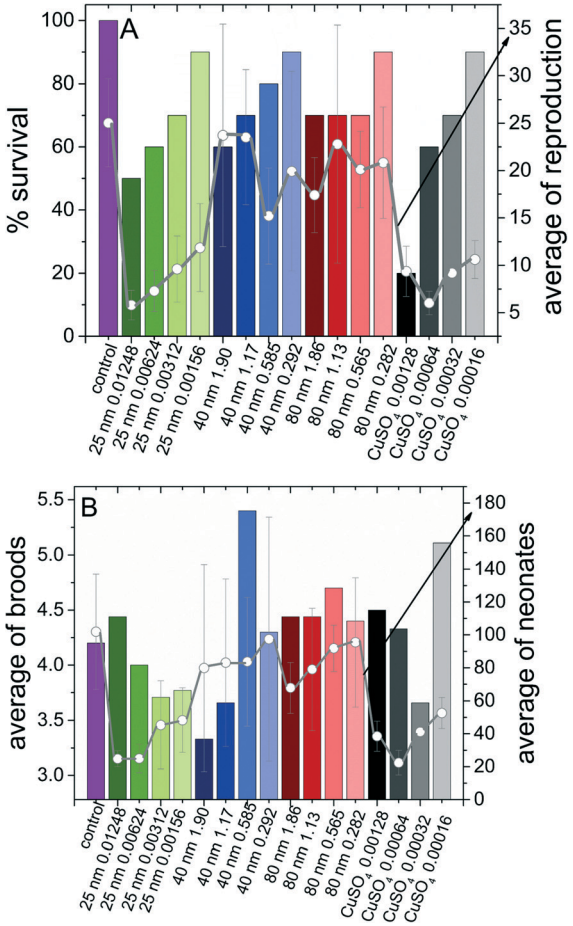

Fig. 3 Effect of differently sized nCuO particles and $CuSO_4$ (A) on the survival (bars), average reproduction (line) and (B) average number of broods (bars) and average number of neonates (line) in the chronic assay with *Daphnia magna*. The raw data is presented in Table S2† and the details about the calculation can also be found in the ESI.† The effects on reproductive output of *D. magna* were more pronounced for 25 nm nCuO and $CuSO_4$.

studies evaluating the effects of several nanoparticles on microbes suggest that the contribution of dissolved ions may be negligible.[47] However, for *D. magna*, the LC_{50} reported in the present study for $CuSO_4$ combined to the solubility of nanoparticles, suggests that dissolved ions may contribute to the toxicity. Heinlaan *et al.* detailed the importance of dissolved ions for the toxic effects of nanoparticles. The toxicity of nano-CuO and bulk CuO was almost identical to that of free Cu^{2+} from $CuSO_4$ (bioavailable Cu). In addition, they showed that the nanoparticles do not have to enter the cells to cause harm, as the contact between the nanoparticle and gut cells may cause changes in the microenvironment such as generation of extracellular ROS.[26]

Fig. 3 shows the results of the 21 day chronic assays. Fig. 3(A) shows the percentage survival (bars) and average reproduction (line), while Fig. 3(B) depicts the average number of broods (bars) and the average number of neonates (offspring) (line). Both the nCuO treatments and the $CuSO_4$ positive control reduced the survival of the daphnids in a dose-related manner. The reduction of survival was more noticeable for the 25 nm nCuO at 0.01248 mg Cu per L and $CuSO_4$ at 0.00128 mg Cu per L, with survival percentages of 50% and 20%, respectively. For the 80 and 40 nm nCuO dispersions, daphnid survival decreased exponentially as a function of concentration between 0.282 and 1.9 mg Cu per L (Fig. S6†). The survival-concentration pattern in the chronic assay was similar to that found for the acute assay, *i.e.* 40 and 80 nm nCuO were less toxic than $CuSO_4$ and 25 nm CuO.

The average reproduction shown in Fig. 3(A) followed the survival trend for the 25 nm nCuO and $CuSO_4$, while for 40 nm and 80 nm nCuO no tendency was observed. The statistical analysis (Fig. S7(A)†) confirmed that the lowest average reproduction was obtained for $CuSO_4$ and 25 nm nCuO, especially at 0.00064 mg Cu per L and 0.01248 mg Cu per L, respectively. The average reproduction (Fig. S7(A)†) did not significantly differ for the 40 and 80 nm CuO.

Fig. 3(B) shows that the average number of broods per daphnid increased following exposure to the nanoparticles while the average number of neonates decreased. Hence, although more broods were produced, the average number of neonates (offspring) was lower.

Table S2 in the ESI† shows that the time to the 1st brood was practically the same for treatments and control. Similar results were reported by Lu *et al.*[31] who found no significant effect on offspring numbers and time to 1st brood at 0.4 μg Cu per L (nCuO < 100 nm). Analogous to the average reproduction, the lowest average numbers of neonates were found for $CuSO_4$ and 25 nm nCuO (Fig. S7(B)†).

The EC_{50} values for the reduction of neonate numbers caused by 40, 80, 25 nm nCuO and $CuSO_4$ were 2.10, 1.95, 0.0018, and 0.00017 mg Cu per L, respectively. According to the Tukey test, the toxicity order was the same as found for the LC_{50}s (Fig. 2(B)). Overall, the statistical analysis showed that the 40 and 80 nm nCuO presented similar effects on the daphnids. And the 25 nm nCuO has a similar toxicity as the $CuSO_4$.

Reduction of the time of first brood and the number of neonates were also reported for *D. magna* exposed for 21 days to nano CuO, ZnO, Au, and TiO_2.[48] In agreement to the present study, a positive relationship between harmful effects and dissolved ion concentrations was reported. Likewise, in 21 days assays, Adam *et al.* reported a reduction of average brood number per female, from 3.9 ± 0.3 in the control to 1 ± 1.4 and 3 ± 0.8 for $CuCl_2$ and CuO, respectively.[23]

The effects of nanoparticles on reproductive parameters may be hypothesized in the light of the available literature. Using a combination of PIXE/RBS/STIM, Pinheiro *et al.* showed the presence of Ti in the eggshell and eggs of *D. magna* exposed for four days to TiO_2 at 2.8 mg Ti per L.[49] Although the limits of detection and lateral resolution imposed by XRF in the present study did not allow detecting Cu in these tissues, one can suppose it might affect the reproduction of the daphnids.

Wang *et al.* investigated the effects of micro/nano-Cu_2O crystals (450–900 nm at 10 μg L^{-1}) on *D. magna*.[50] The time to the first brood and offspring growth were evaluated during 30 days of exposure. In agreement to the present study, the time to first brood was affected by the treatments (2 days delayed for daphnids exposed to Cu_2O vertex-truncated octahedron shapes). All six micro/nano-Cu_2O crystals reduced the length of individuals compared to the control. In the most extreme case, the treatment reduced the length by 48.7%.

To better understand the biological effects of the nCuO particles on the daphnids, their chemical reactivity was evaluated in terms of H_2O_2 decomposition. The O_2 evolution curves showed that 25 nm nCuO was the most reactive particle in both deionized water and culture medium for 1000 mg Cu per L (Fig. S8(A) and (B)†). At concentrations corresponding to the LC_{50}s, the amount of O_2 produced by the 25 nm nCuO was lower than for the other nCuO particles and $CuSO_4$ (Fig. S8(C)†), which may be justified by its low concentration. The reactivity of $CuSO_4$ was lower in culture medium than in water, which might be related to its chemical availability since a fraction of the Cu ions was precipitated. The amount of O_2 produced by 40 and 80 nm nCuO was similar, regardless of the liquid media. In the literature there is no consensus as to whether toxicity is caused by the dissolved copper or by nanoparticles.[7,30,31] Since the trend in H_2O_2 decomposition was in line with the results of the toxicological assays, it seems that the redox reactivity of Cu ions and nanoparticles may play a role in the mechanism of toxicity.

Spatial distribution of Cu in dead daphnids

Fig. 4 presents quantitative maps revealing the spatial distribution of Cu in the *D. magna* exposed to nCuO and $CuSO_4$ during the acute assays. The local concentration is expressed as μg of Cu per cm^2. The concentration of Cu in the spots varied from 2.9 up to 255 μg cm^{-2}.

Instead of appearing homogenously distributed along the carapace, Cu was found as spots in the gut region. This suggests that Cu was stored in the soft parts of the daphnids, such as the intestine and appendages. These findings are in agreement with scanning electron microscopy results reported by Wang *et al.*, 2015.[50]

The daphnids exposed to $CuSO_4$ showed a ruptured carapace, which helps explaining the low internal concentrations since the Cu concentrated inside may have leached out due to these ruptures. It is worth highlighting that the fixation procedure did not alter the elemental spatial distribution, considering a lateral resolution of tens of micrometres.

Fig. 5 shows the spatial distribution of Cu in the body of adult daphnids exposed for 21 days to (A) 80 nm nCuO at 1.86 mg Cu per L and (B) 40 nm Cu at 1.90 mg Cu per L. Similar to the acute assays, the Cu was mainly accumulated in

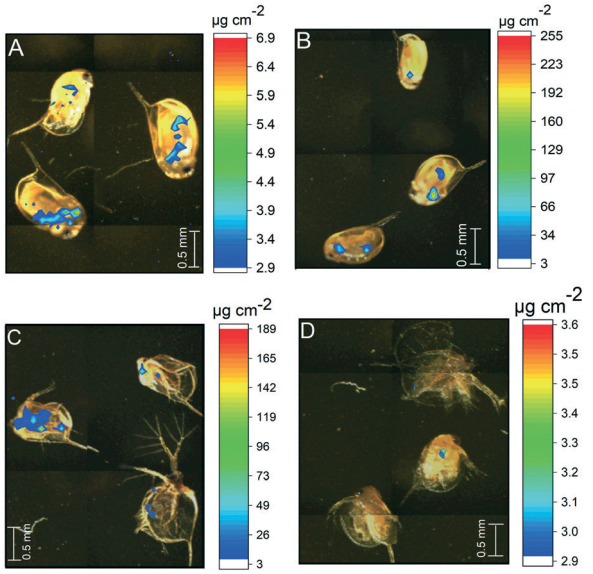

Fig. 4 Quantitative spatial distribution of copper in the body of *Daphnia magna* neonates exposed for 48 h to nCuO and $CuSO_4$ at concentrations killing all organisms (LC_{100}) in the acute assays. (A) Neonates exposed to 25 nm nCuO at 0.15 mg Cu per L, (B) neonates exposed to 40 nm nCuO at 4.5 mg Cu per L, (C) neonates exposed to 80 nm nCuO at 16 mg Cu per L, (D) neonates to $CuSO_4$ at 0.32 mg Cu per L. Cu accumulation hotspots were found in the intestine and soft tissues.

the intestine and soft parts. We did not find any other reports comparing the spatial distribution of the same nanoparticles within neonates and adults. Due to the thickness of adult *D. magna*, it was not possible to transform the XRF counts into concentrations. Nevertheless, the number of Cu-Kα counts is directly proportional to the Cu content. Thus, the brighter points in Fig. 5 indicate Cu hotspots. It was not possible to detect XRF signals for Cu in the daphnids exposed to 25 nm Cu and $CuSO_4$.

In agreement to our findings, CdSe/ZnS quantum dots,[51] C60 (17–23 nm)[46] and CuO nanoparticles (30 and 30–50 nm)[7,46] were shown to be concentrated in the gut of daphnids.[7,46,52] Adult *D. magna* exposed to 0.12 mg L^{-1} dissolved Zn (from quantum dots) for one week accumulated Zn mainly in the gut region and eggs, where concentrations reached 30 μg cm^{-2}.[53,54]

In agreement to the present study, SEM images showed that micro/nano-Cu_2O tended to agglomerate inside the gut. This suggests that damage can be caused by low concentrations of dissolved metal ions in the gut and that the concentration of soluble copper is higher at the octahedron shape of micro/nano-Cu_2O.[50]

The literature describes several mechanisms by which particles and ions can harm or compromise the health of *D. magna*, including intracellular effects, mobility and obstruction effects.

Daphnids are filter feeders. A 2–3 mm wide *D. magna* can filter up to 400 mL of liquid medium per day.[55] Upon filtration, particles can get trapped inside the organisms and ions can be taken up into cells by endocytosis.[23,55] Ions can also be absorbed by ion channels or by ionic pumps located on cells of the intestine.[56,57] In *Gammarus pulex* the excess of ions and particles was shown to compromise the osmoregulation process generating a metabolic expense in the organism.[31] Specifically, Cu ions can reduce sodium absorption.[46]

Nanoparticles can adhere to the carapace and therefore hinder the mobility of the daphnid, leading to a change in its swimming behavior.[31] The movement of appendages in daphnids is constant and occurs even in the absence of food. This movement, besides aiding in the filtration of particles, acts in the circulation of the aqueous medium in the carapace and facilitates respiration. Movements can also transfer particles located in the appendages towards the intestine.[52] Another study reported that diamond nanoparticles can accumulate within the gastrointestinal tract and block the absorption of nutrients by the intestinal cells in *D. magna*.[58] Deficiency in nutrient absorption can lead to slow growth and reduced fecundity.

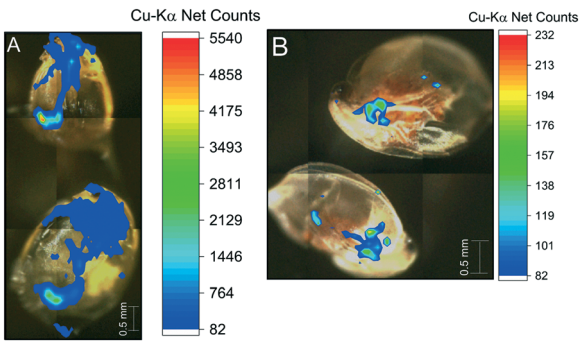

Fig. 5 Spatial distribution maps of copper in the body of *Daphnia magna* exposed for 21 days to (A) 80 nm nCuO at 1.86 mg Cu per L and (B) 40 nm nCuO at 1.90 mg Cu per L. Cu accumulation hotspots were found in the intestine and soft tissues.

μ-XANES chemical speciation of Cu within Cu hotspots

The chemical speciation of the Cu taken up by the daphnids was investigated by measuring XANES on the Cu K edge.

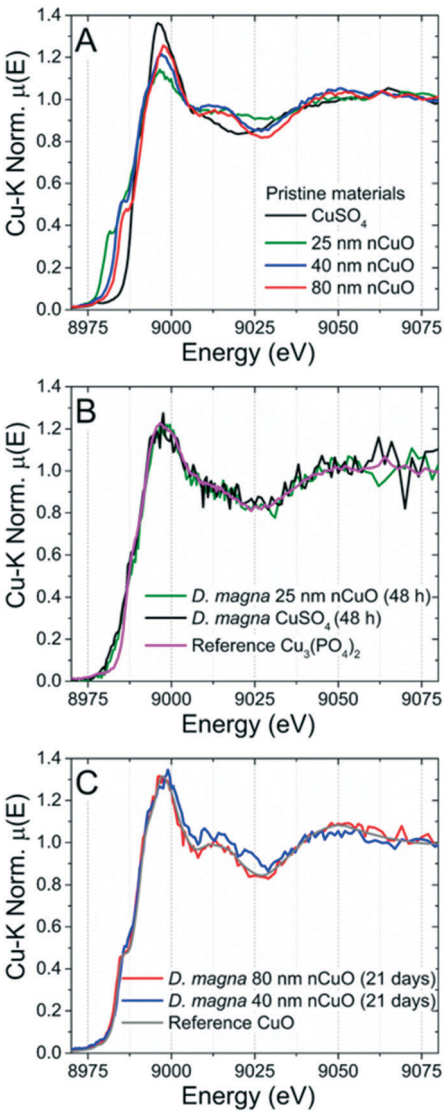

Fig. 6 Spectra generated by XANES analysis of (A) pristine $CuSO_4$ and 25 nm CuO materials, (B) of *Daphnia magna* exposed for 48 h to $CuSO_4$ and nCuO 25 nm at 16 mg Cu per L, and (C) of *D. magna* exposed for 21 days to 80 and 40 nm at 1.86 and 1.90 mg Cu per L, respectively. $CuSO_4$ and 25 nm CuO were transformed into a compound chemically similar to $Cu_3(PO_4)_2$, while 40 nm and 80 nm CuO remained mainly as CuO.

Fig. 6(A) shows the XANES spectra for the nCuO and $CuSO_4$ pristine materials used in the exposure assays. Fig. 6(B) shows that the μ-XANES spectra of daphnids exposed to 25 nm nCuO and $CuSO_4$ for 48 h are similar to that of $Cu_3(PO_4)_2$. This indicates that 25 nm nCuO and $CuSO_4$ were converted into $Cu_3(PO_4)_2$. The $Cu_3(PO_4)_2$ inside *D. magna* possibly was formed after reaction with the phosphate buffer used to preserve the carapace of the daphnids, so copper analysed was inside the daphnids. More research is needed to address the possible changes occurring during sample preparation. Fig. 6(C) shows that the Cu chemical neighbourhood inside daphnids exposed for 21 days to 40 nm and 80 nm nCuO remained as CuO.

The μ-XANES findings agreed with the results of the H_2O_2 decomposition tests. Since the 40 and 80 nm nCuO were not transformed, it confirms their lower reactivity, and therefore availability, compared to 25 nm nCuO and $CuSO_4$.

Using XANES, Sávoly *et al.* demonstrated that $CuSO_4$ was biotransformed in the nematode *Xiphinema vuittenezi* exposed to 1 mmol L^{-1} $CuSO_4$.[59] They found Cu binding species similar to glycine, glutamate acid and histidine.

Ivask *et al.* employed XANES to investigate the chemical environment of Cu in a T-lymphocyte cell line exposed to a non-toxic concentration (2 μg Cu per mL) of 15 nm CuO and $CuSO_4$.[60] Different from our results, they reported Cu-cysteine (55–58%) as the major species for both treatments. In addition, minor fractions of Cu-histidine (11–20%), Cu-citrate (4–11%), and CuO (15–22%) were detected. The same Cu species, but in different proportions, were found for the treatments in culture medium without cells. Copper II oxide was found even in the cells and culture medium exposed to $CuSO_4$.

Conclusions

The combination of μ-XANES and μ-XRF showed that Cu is accumulated in the intestine of daphnids as $Cu_3(PO_4)_2$ and Cu oxide, the chemical form depending on the type of nanoparticle. Since 25 nm nCuO and $CuSO_4$ were more reactive, low concentrations were used in the acute (0.031–0.32 mg Cu L^{-1}) and chronic (1.248×10^{-3}–1.6×10^{-4} mg Cu per L) assays and therefore less Cu was found within the *D. magna*. The highest reactivity of 25 nm nCuO and $CuSO_4$ might have favored their conversion into $Cu_3(PO_4)_2$.

On the other hand, 40 and 80 nm nCuO presented higher LC_{50} and EC_{50} values, in turn more Cu was absorbed by the organisms making their detection by μ-XRF easier (Fig. 4). Since μ-XANES showed that the nCuO did not undergo biotransformation, the exhibited toxic effects might mainly be related to physical hindrance phenomena. Obstruction of the intestine seems to be at least partially responsible for the biological effects.

An imbalance of dissolved Cu could also have induced toxic effects, with a similar mechanism of action as that of the ionic Cu dosed as $CuSO_4$. The Cu ions could be admitted within the cells and the toxicity mechanism would be intracellular. Copper ions may pass through the membrane (cells) and enter into the cytoplasm. This passage can generate reactive oxidative species (ROS) which leads to oxidative stress; both are toxicological mechanisms for cell damage induced by nanoparticles.

The highest toxicity exhibited by the 25 nm nCuO might be related to their core-shell structure that consisted of a Cu metal core covered by oxidized Cu_2O and CuO layers. The highest toxicity might be related also to their higher solubility that increased Cu availability in the medium. Besides hindering the proper absorption of nutrients, it is likely that the 25 nm nCuO might have promoted oxidation or reduction of biomolecules that negatively affected the daphnids.

Altogether, the results show that in addition to particle size, composition and concentration, the structure might influence the toxicological behavior and the environmental impact of CuO nanoparticles.

Conflicts of interest

The authors declare that there are no conflicts to declare.

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